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Degradation of milk-based bioactive peptides by yogurt fermentation bacteria*

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Abstract

Aims: To analyse the effect of cell-associated peptidases in yogurt starter culture strains *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) and *Streptococcus thermophilus* (ST) on milk-protein-based antimicrobial and hypotensive peptides in order to determine their survival in yogurt-type dairy foods.

Methods and Results: The 11mer antimicrobial and 12mer hypotensive milk-protein-derived peptides were incubated with mid-log cells of LB and ST, which are required for yogurt production. Incubations were performed at pH 4·5 and 7·0, and samples removed at various time points were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). The peptides remained mostly intact at pH 4·5 in the presence of ST strains and moderately digested by exposure to LB cells. Peptide loss occurred more rapidly and was more extensive after incubation at pH 7·0.

Conclusions: The 11mer and 12mer bioactive peptides may be added at the end of the yogurt-making process when the pH level has dropped to 4·5, limiting the overall extent of proteolysis.

Significance and Impact of the Study: The results show the feasibility of using milk-protein-based antimicrobial and hypotensive peptides as food supplements to improve the health-promoting qualities of liquid and semi-solid dairy foods prepared by the yogurt fermentation process.

Introduction

Over the years, scientists have sought methods to impart potential health benefits to dairy foods. There have been numerous reports of milk-based proteins and peptides that have hypotensive (Gobbetti *et al.* 2002; FitzGerald *et al.* 2004) or antimicrobial properties (Hoek *et al.* 1997; Minervini *et al.* 2003) that are released upon proteolysis of whey proteins (Meisel and Bockelmann 1999). A 12 amino acid fragment of α_{s1}-casein with the sequence FFVAPFPEVFGK has an inhibitory effect on the angiotensin-I-converting enzyme (ACE) (Maruyama *et al.* 1987; Karaki *et al.* 1990; Townsend *et al.* 2004) and therefore may potentially reduce peripheral blood pressure and be an effective antihypertension treatment (Ondetti *et al.* 1977). A few food products have been developed that have utilized milk-derived ACE-inhibitory peptides, including Ameal S, a tablet-form sour milk product on the market in Japan (Nakamura *et al.* 1995a,b; Hayes *et al.* 2007). To date, these types of antihypertensive compounds have not been incorporated into any available fermented dairy products like cheese or yogurt.

Lactoferrins are mammalian iron-binding glycoproteins that are known to exhibit broad spectrum antimicrobial activity (Strom *et al.* 2000). Enzymatic digestion of bovine (Tomita *et al.* 1991) and human (Bellamy *et al.* 1992) versions of these proteins results in smaller peptides (known as lactoferricins) that exhibit antimicrobial activity. Synthetic and enzymatic truncations of the full lactoferrin proteins have yielded sequence domains in the original molecule that are required for antimicrobial activity (Hoek *et al.* 1997; Strom *et al.* 2000, 2001). Digestion of bovine lactoferrin with pepsin releases an 11mer peptide (RRWQWRMKKLG) with potent antimicrobial activity (Kang *et al.* 1996) that may be commercially applicable in the food industry as a safe and

effective biopreservative (Papagianni 2003; Chatterjee *et al.* 2005).

Because the peptides described here are both derived from milk proteins, it may be possible to utilize the 12mer antihypertensive peptide or the 11mer antimicrobial lactoferrin fragment in dairy food products prepared by a yogurt fermentation process. The major limitation in this application lies in the presence of the starter cultures *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) that are needed for milk fermentation. However, the cell-associated peptidases of these lactic acid bacteria (LAB) may inactivate the bioactive peptides by hydrolysing them to amino acids (Gilbert *et al.* 1996; Siezen 1999; Fernandez-Espla *et al.* 2000; Courtin *et al.* 2002).

In this report, we describe the changes in the structural integrity of the milk-protein derived antimicrobial 11mer and the hypotensive 12mer peptide after exposure to live cells of selected LAB under conditions simulating yogurt fermentation.

Materials and methods

Bacterial strains, growth media and resting cell assays

All bacterial strains used were from an in-house collection. The ST strains ST101, ST108 and ST119 were grown in TYL (Tryptone–Yeast extract–Lactose) broth (Somkuti and Steinberg 1986), while LB strains LB6, LB11 and LB20 were grown in deMan, Rogosa and Sharpe medium (MRS; Becton Dickinson, Sparks, MD, USA). All ST and LB cultures were propagated at 37°C.

The 11mer antimicrobial and the 12mer hypotensive peptides were synthesized by EZBiolab, Inc. (Westfield, IN, USA). The sensitivity of the peptides to putative cell-bound peptidase enzyme activity was tested by using resting cell suspensions of ST and LB strains prepared from mid-exponential phase (OD_{660} = 0·5) cultures (20 ml per tube). The cultures were centrifuged at 10 000 *g* for 15 min at 4°C. Cell pellets were washed twice in 10 mmol l^{-1} phosphate buffer (pH 4·5 or 7·0) and reconstituted in 1 ml of 10 mmol l^{-1} phosphate buffer, representing a 20-fold increase in cell density. Cell suspensions were incubated with the 11mer or 12mer peptides at 37°C.

RP-HPLC time studies

The 11mer antimicrobial and 12mer hypotensive peptides were used at 500 μg ml^{-1} concentration in assays to test their sensitivity to LAB. Aliquots from these assays were removed and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) carried out on an Agilent 1200 instrument using a Vydac C18 peptide column (5 mm × 4·6 mm × 250 mm). The following conditions were utilized for the RP-HPLC runs: flow rate of 1 ml min^{-1} at 25°C; solvent A is 0·1% trifluoroacetic acid (Sigma-Aldrich) in Millipore water; solvent B is 0·086% trifluoroacetic acid in acetonitrile (Mallinckrodt Baker Inc., Phillipsburg, NJ). Analysis was performed using a linear elution gradient of 5–100% solvent B (95–0% solvent A) over 50 min for all samples. Absorbance was recorded at 220 nm using Agilent ChemStation® HPLC software. Peaks corresponding to peptide degradation products were collected using an Agilent 1200 series fraction collector and analysed by matrix-assisted laser desorption ionization–time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS), employing an Applied Biosystems 4700 Proteomics Analyzer – MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA). Mass spectral data were further analysed using the FindPept tool provided by the ExPASy Proteomics server (http://www.expasy.ch). The amount of 11mer or 12mer peptide remaining after exposure to nonproliferating bacterial cells was calculated using a standard curve constructed for each peptide by measuring the absorption of stock solutions (0–25 μg ml^{-1}) at 220 nm.

Results

Sensitivity of 11mer peptide RRWQWRMKKLG to ST

The antimicrobial 11mer peptide was incubated with cells of ST strains ST101, ST108 and ST119 to determine the extent of proteolytic breakdown as a function of time. RP-HPLC analysis of samples of the 11mer peptide taken during exposure to cell suspensions of ST108 at pH 4·5 indicated insignificant degradation over the span of 4 h (Fig. 1a). Furthermore, calculation of residual peptide concentration showed that even after 4 h exposure peptide loss varied from 1% to 13%, depending on the ST strain used (Table 1). However, incubation of the 11mer peptide at pH 7·0 indicated that a significant portion of the peptide was already lost after 30 min of exposure (Fig. 1b) and that proteolysis was complete within 2 h (Fig. 1c). HPLC analysis of samples treated with nonproliferating cells of strains ST101 and ST119 yielded similar results under the same conditions (data not shown). Calculation of peptide concentrations confirmed a greater peptide loss that occurred more rapidly at pH 7·0 (Table 1).

RP-HPLC analysis of samples showed that treatment of the 11mer peptide with cell suspensions of LB strains resulted in varying degrees of proteolysis at both pH 4·5 and 7·0. The extent of proteolysis was time dependent, and the amounts of 11mer peptide remaining after exposure at pH 4·5 and 7·0 are shown in Table 1. The 11mer

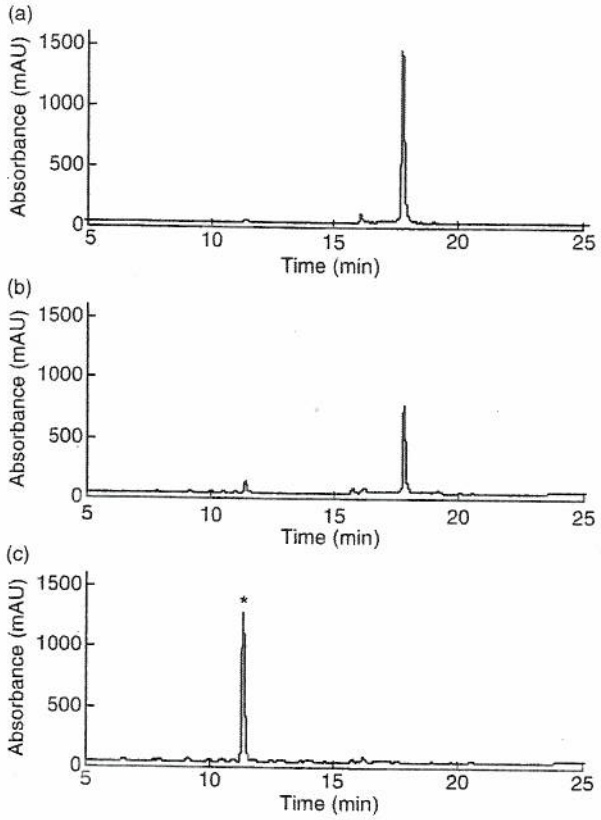

Figure 1 C18 RP-HPLC chromatograph of 11mer peptide with ST108 cells (a) before and after 4 h treatment, pH 4·5; (b) after 30 min, pH 7·0; and (c) after 2 h, pH 7·0 [(*) designates the peak collected for MS analysis].

Table 1 The effect of pH on the hydrolysis of 11mer antimicrobial peptide by *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) strains

Strain (pH)	Incubation time (h)	% Peptide remaining
Control-no bacteria (7·0)	0	100
ST101 (4·5)	4	91
ST108 (4·5)	4	>99
ST119 (4·5)	4	87
ST101 (7·0)	1	15
ST108 (7·0)	0·5	48
ST108 (7·0)	2	<0·1
ST119 (7·0)	1	4
LB6 (4·5)	3	>99
LB11 (4·5)	2	84
LB20 (4·5)	1	70
LB20 (4·5)	4	35
LB6 (7·0)	1	5
LB11 (7·0)	0·5	<0·1
LB20 (7·0)	0·5	<0·1

peptide remained stable in the presence of LB6 at pH 4·5 even after 3 h, while exposure to strain LB11 for 2 h or to strain LB20 for 4 h resulted in 16% and 65% loss of the peptide, respectively. Time studies with LB strains at pH 7·0 showed that proteolysis of the 11mer peptide was more extensive and that degradation of the peptide by LB11 was nearly complete after 30 min (Table 1). Similar results were obtained with strains LB6 and LB20.

Mass spectrometric analysis of degradation products produced by incubation of the 11mer peptide with both LB and ST cells at pH 7·0 revealed that the major product present after incubations in all cases was the peptide WQWRM (m/z = 806·1, Fig. 1c). This fragment corresponds to residues 3–7 of the intact peptide, RRWQWRMKKLG (MS data not shown). Masses corresponding to sequential loss of the Arg1 (m/z = 1388) and Arg2 (m/z 1247 with methionine oxidation) as well as a peptide consisting of residues 3–8 with cleavage after Arg2 and Lys8 (m/z 953 with methionine oxidation) were also observed.

Sensitivity of 12mer peptide FFVAPFPEVFGK to ST and LB cells

RP-HPLC analysis of the antihypertensive 12mer peptide following treatment with nonproliferating cells of strains ST101, ST108 and ST119 at pH 4·5 showed a moderate reduction in peptide concentration (Fig. 2a), with 66%, 70% and 78% of the peptide remaining intact, respectively,

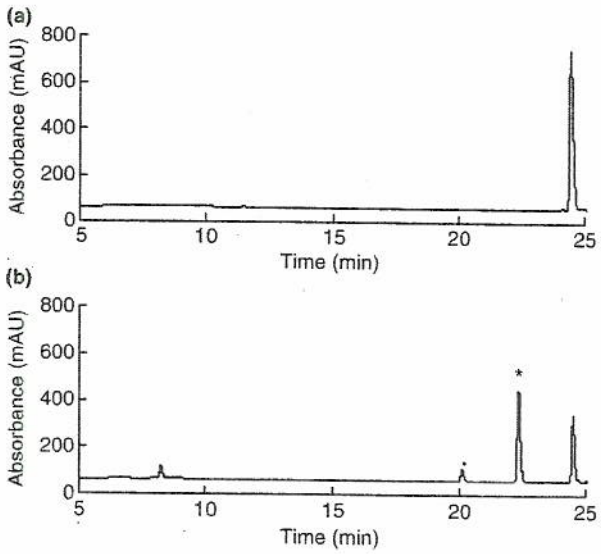

Figure 2 Chromatograph of 12mer peptide incubated with ST119 cells before and after 4 h (a) at pH 4·5 and (b) at pH 7·0 [(*) designates the 2-to-12 truncated degradation product and (■) corresponds to the 3-to-12 residue peptide].

Table 2 The effect of pH on the hydrolysis of 12mer hypotensive peptide by *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB) strains

Strain (pH)	Incubation time (h)	% Peptide remaining
Control-no bacteria (4·5)	0	100
ST101 (4·5)	4	66
ST108 (4·5)	4	70
ST119 (4·5)	4	78
ST101 (7·0)	4	22
ST108 (7·0)	4	18
ST119 (7·0)	4	33
LB6 (4·5)	4	75
LB20 (4·5)	4	74
LB11 (4·5)	4	69
LB6 (7·0)	4	34
LB11 (7·0)	1	<1
LB20 (7·0)	3	<1

even after 4 h of incubation (Table 2). Although exposure of the 12mer peptide to ST strains at pH 7·0 showed significant proteolytic degradation of the peptide (Fig. 2b), leading to a 78%, 82% and 67% loss in the presence of ST101, ST108 and ST119, respectively (Table 2), these losses were lower than that observed with the 11mer peptide (Table 1), suggesting that the 12mer hypotensive peptide was more resistant to proteolysis by ST peptidases. Similar results were obtained with the 12mer peptide when incubated with the LB cells. At pH 4·5, HPLC analysis indicated only limited degradation during the first 60 min, and no further observable peptide loss occurred after 2, 3 or 4 h of incubation (data not shown). After 4 h, the loss of 12mer peptide was between 25% and 30%, depending on the LB strain used (Table 2). At pH 7·0, the 12mer peptide was more resistant to proteolytic degradation by LB6, with *c.* 35% of the peptide remaining intact even after 4 h of incubation, while exposure to LB11 and LB20 cells resulted in nearly total loss of the peptide (Table 2).

Analysis of the degradation products isolated after incubation of the 12mer with ST and LB cells at pH 7·0 revealed masses corresponding to sequential cleavages of the first few residues from the N-terminus of the peptide. The incubations with the ST cells yielded the 2-to-12 residue peptide FVAPFPEVFGK as the major degradation product with the 3-to-12 residue peptide VAPFPEVFGK also present in smaller quantities (Fig. 2b). Samples of the LB incubations contained many more peaks whose masses corresponded to the 2-to-12 and 3-to-12 truncated peptide as well as the 4-to-12 and 5-to-12 sequences.

The concentration of 11mer and 12mer peptides were also determined in samples stored for 10 days at 4°C following incubation with ST and LB cells at pH 4·5. The results obtained were no different from that shown in Tables 1 and 2, indicating the stabilizing effect of refrigeration that apparently prevents or minimizes additional peptide loss caused by proteolysis.

Discussion

Yogurt fermentation takes place in distinct phases that are influenced by two synergistically interacting bacterial cultures, ST and LB (Radke-Mitchell and Sandine 1986). The early phase of the fermentation (pH 6·5–5·5) is governed predominantly by the ST component, which produces growth factors that stimulate growth of LB at lower pH values (Vinderola *et al.* 2002). Both cultures produce lactic acid that lowers the pH to 4·25–4·5, which is characteristic of the final product (Shah 2000). To consider the introduction of any potentially therapeutic or beneficial compound into this system, it is necessary to examine the influences of these two strains of bacteria on the supplemented material at both low and high pH (4·5 and 7·0).

The presence and activities of cell-associated proteases in both ST and LB strains have been well documented (Siezen 1999). Lactobacilli are reported to have high cell surface proteinase activity (Gilbert *et al.* 1996), while most strains of ST have little to no cell-associated protease activity (Fernandez-Espla *et al.* 2000). This is evidenced by comparing the degradation products obtained from incubations with the ST and the LB cells at pH 7·0. The overall digestion pattern of the 11mer peptide was identical between the ST and LB cells, with evidence of specific cleavage at the basic arginine and lysine residues within the peptide. However, digestion of the 12mer peptide showed that overall degradation by the ST cells was less extensive, resulting in fewer peptide fragments, than in the case of LB cells.

Furthermore, digestion of the 12mer peptide by the LB cell-associated peptidases appears to be inhibited once a proline residue is encountered, resulting in the smallest fragment observed corresponding to the sequence PFPEVFGK. This suggests that it may be possible to limit overall proteolysis through mutation of an earlier residue or introduction of an N-terminal proline that may inhibit the peptidases, providing that bioactivity is not compromised.

The addition of bioactive peptides at the start of yogurt fermentation by ST and LB strains may result in extensive degradation because of the cumulative effect of LAB peptidase/protease activities before the fermentation is complete, thus limiting the commercial utilization of milk-based antihypertensive and antimicrobial peptides as bioactive food supplements. However, our results show that application of compounds such as the bioactive 12mer (hypotensive) or the 11mer (antimicrobial) peptide

in fermented dairy foods may be possible if they are added after the pH level is lowered because of vigorous cell growth and lactic acid production by the ST and LB starter cultures. The integrity of the peptides is apparently protected at or below pH 4·5 and a significant portion of the peptides remains intact even after 4 h of contact with cells of ST or LB strains. Because ST and LB strains are always used in combination as starter cultures in yogurt fermentation, care should be taken to pair strains with moderate levels of peptidase/protease activity (see Tables 1 and 2). This would ensure that even the cumulative effect of ST and LB peptidase/protease activities would not cause a total elimination of the bioactive peptides when used as health-promoting or food-protective supplements in fermented dairy foods.

Although the results of work described here suggest that the addition of bioactive peptides should take place at or near the end of fermentation (pH 4·25–4·5), this may not be possible under conventional yogurt manufacturing conditions without disturbing or destroying the integrity of the coagulum. However, health-promoting bioactive peptides found in milk proteins may be conveniently blended into drinkable yogurt-like products that have significantly increased in popularity and consumer acceptance.

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